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Chapter 6

Phylogeny and evolution

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Introduction

Nematodes originated during the Precambrian explosion over 500 million years ago (Wray *et al*., 1996; Ayala & Rzhetsky, 1998; Rodriguez-Trelles *et al*., 2002). The phylogenetic position of the Nematoda relative to other metazoans has historically been one of contention. Originally circumscribed within the Vermes Linnaeus, 1758 and later the Aschelminthes Grobben, 1910 (Claus & Grobben, 1910), the Nematoda are now believed to belong to a clade of moulting animals, the Ecdysozoa (Aguinaldo *et al*., 1997), and share a most recent common ancestor with arthropods, kinorhynchs, nematomorphs, onychophorans, priapulids and tardigrades.

ORIGINS OF ENTOMOPATHOGENIC NEMATODES (EPN)

Entomopathogenic nematodes of the Steinernematidae and Heterorhabditidae are not monophyletic, but likely began independently to explore biotic relationships with arthropods and Gram-negative enteric bacteria (Enterobacteriaceae) by the mid-Palaeozoic (approximately 350 million years ago) (Poinar, 1993). Their origins were probably not synchronous and the ages of their respective lineages appear to be significantly different. Evidence for the disparate origins and relative age of these Families is illustrated in Figure 240. Assuming even a somewhat sloppy molecular clock, the long branch lengths of *Steinernema*, both within the genus and relative to its most recent common ancestor, imply that it has been evolving independently of other nematode lineages for a longer period of time than *Heterorhabditis*. This could explain, in part,

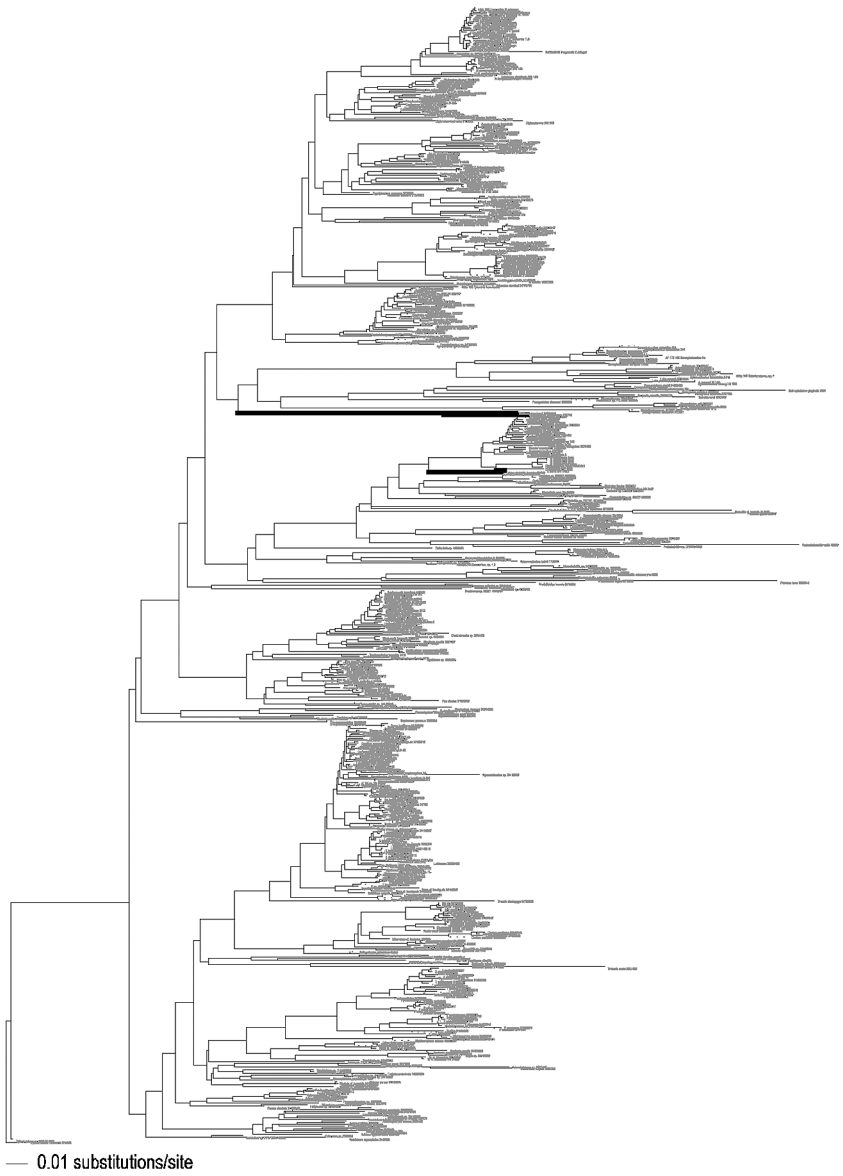

Fig. 240. *Phylogenetic tree of 434 representative taxa from the Rhabditida, Plectida and Monhysterida. The tree was generated from 18S rDNA sequences by the neighbour joining BioNJ algorithm using log determinant transformed distances in order to account for rate and nucleotide usage heterogeneity among lineages. EPN branches are in bold.*

the differences in species richness between the two genera (*i.e.*, *Heterorhabditis* has had less time to generate species). However, if EPN in general have been coevolving with their insect hosts for 350 million years, why do we not see numbers of entomopathogenic nematodes equal to the species richness of their insect hosts? Even if the number of species of insect hosts were whittled down to only those that have a soil-borne larval stage, the host-parasite species deficit would still be tremendous.

ORIGINS OF EPN SPECIES

Little is known of the modes of speciation that have produced the extant biodiversity of EPN. Because most EPN sister species appear to be (or have been) geographically isolated (Hominick *et al.*, 1996; Hominick, 2002), vicariant allopatric speciation, in most cases followed by dispersal, is the most likely mode of speciation to explain their distribution (Lynch, 1989). There are several hypothetical reasons as to why we might predict that there should be more species of EPN than have been described to date. First of all, there appears to be a correlation between species size and the probability of extinction (Jackson, 1974; Jablonski, 1987). Species with large range sizes, such as bison and woolly mammoths, require more resources and space and, therefore, have a lower probability of speciating and a higher probability of extinction. By contrast, animals such as EPN that have a small body size may be able to partition more niche space per unit area (Hutchinson & MacArthur, 1959; Morse *et al.*, 1985). Therefore, because nematodes have small spatial requirements, they should have higher speciation rates and greater niche filling or niche partitioning opportunities. Just such partitioning is evident in studies of habitat preference (Sturhan, 1999) and could be maintained phylogenetically (Spiridonov *et al.*, 2004).

Fig. 240. *(Continued). The upper bold lines depict the lineages of* Steinernema carpocapsae *and* S. glaseri *relative to their most recent common ancestor. The lower bold lines illustrate the branching point of* Heterorhabditis marelatus *and* H. bacteriophora *relative to their most recent common ancestor. Note the long branch length of the steinernematid lineage relative to the heterorhabditid lineage, and the long branch lengths of the steinernematid species relative to the heterorhabditid species. The multiple sequence alignment for this clade was retrieved from NemaTOL (nematol.unh.edu).*

However, nematodes could have high rates of dispersal, as evidenced by the inter- and transcontinental geographic ranges of many species (Adams *et al*., 2006); their effective range requirements could be vast, effectively decreasing the probability of speciation. In the case of EPN, their small body size, coupled with an association with mobile insect hosts, could facilitate dispersal and gene flow across expansive geographic ranges, resulting in retarded speciation rates or extinction probabilities comparable to those of megafauna (but see Blouin *et al*., 1999).

Additional explanations for the patterns of EPN biodiversity are legion but the rate of molecular evolution can probably be ruled out, as it has been shown that for multiple genes nematodes evolve at anomalously high rates relative to other metazoans (*e.g.*, Wray *et al*., 1996). Rather, mechanisms of host specificity and virulence may exert greater influence on the origin of species (or lack thereof) in these genera than other factors (Figs 241, 242). For example, if the nematode depends on the survival or extended mobility of its insect host to vector it to other potential hosts (offspring, mates, *etc*.), then there will be strong selection pressure to reduce virulence because if the host is killed too quickly the nematode will have extinguished the resources or transmission mechanisms required to continue its life cycle (net positive differential reproduction). Reliance on the host as a vector should favour nematode-insect host specificity because the nematode must closely track the evolutionary trajectory of its host. According to this scenario, as the insect speciates, the nematode species must also adapt to changes in its host that involve the nematode's ability to maintain a symbiotic relationship. This would result in tight host-tracking and a longer-term relationship between an individual nematode and its insect host that leads to decreased opportunities for gene flow among other members of its population that have a broader host range. Such a scenario promotes evolutionary fidelity between the nematode and insect host such that if the insect has a high rate of speciation, the nematode must also speciate rapidly as it tracks its host over time. Thus, entomogenous nematodes with low virulence are predicted to have simultaneously narrower host ranges, lower cophylogenetic fidelity, lower rates of gene flow, and comparable rates of speciation relative to those of their insect host. Under this scenario, we would expect strong selection for the evolution of avirulence. Production of toxins or reproduction would increase only to the point that it hindered host mobility or longevity (Fig. 241).

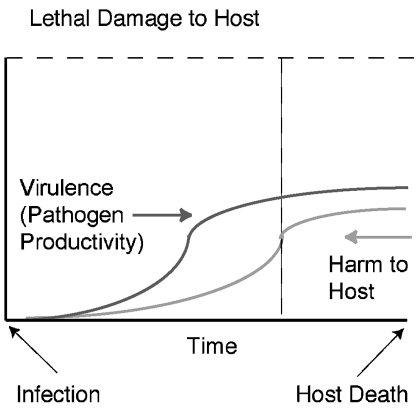

Fig. 241. *Selection between hosts, where inclusive fitness is a function of host mobility/survival. When insect host mobility or longevity is required for successful completion of the nematode life cycle, EPN/B virulence, as measured by either toxin production or reproduction, increases at a maximal rate to establish infection and reproduction, but tapers off as it begins to influence host survival. Such a scenario favours the evolution of increasing avirulence, narrow host ranges, increased nematode-insect host fidelity (cophylogeny), decreased gene flow among nematode populations, and increased rates of speciation.*

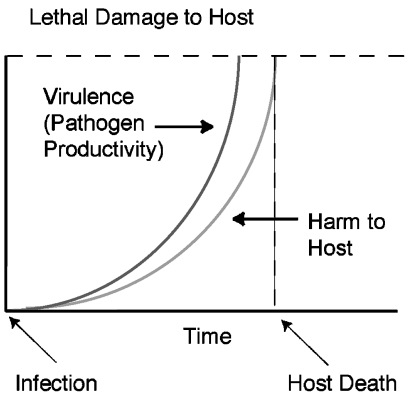

Fig. 242. *Selection within hosts, where inclusive fitness is a function of the rate of reproduction in the host. Competition for resources among different pathogens in an insect host can lead to selection pressures that favour increased virulence. Accordingly, virulence, as measured by toxin production or reproduction, increases at a maximum rate culminating with the rapid death of the host insect. Such a scenario favours the evolution of increasing virulence, broad host ranges, decreased host fidelity (cophylogeny), increased gene flow, and decreased rates of speciation.*

Relative to other families of nematodes, or clades for that matter (Figs 240, 243), the Steinernematidae and Heterorhabditidae contain relatively few species. This may be due, in part, to the discrepancy between the number of EPN species that currently exist and the number of EPN species that have been described (Liu *et al*., 2000). Taxonomic efforts aside, it may be that the highly virulent nature of these nematode-bacterium complexes works to retard speciation in these nematodes. For example, the rapid rate at which the bacterium kills its insect host may have evolved in response to the highly competitive environment inside the host cadaver where bacterial colonies are under strong selection pressure to be as virulent as possible. Considering that the insect represents a rich carbon resource for soil microbes and the tremendous diversity of soil microbes positioned to utilise it, individual bacterial colonies that can get into the insect, utilise cadaver resources and replicate the fastest have the greatest chance of being vectored into the next insect by the emergent IJ. Under these conditions, natural selection for high rates of within-host competition results in virulence which continually increases to an optimum that is tempered only by the rate at which the nematode can evolve tolerance to the bacterial toxins (Fig. 3). The rapid death of the host obviates its ability to vector the nematodes (and their endosymbiotic bacteria) back to insect host nests, breeding sites, mates or offspring. The result is that as the infective juveniles emerge from their host's cadaver, they may be equally, if not more, likely to encounter a potential insect host that is a different species to the one from which they just emerged. Thus, the increase in virulence that is generated by competition within the host reinforces the requirement for a broad host range, and *vice versa*. Similarly, high virulence and broad host ranges decrease the requirement for nematodes to establish host-specific relationships. Subsequently, as hosts speciate, there is no selective pressure on the nematodes to do the same. Thus, nematodes that are highly virulent and have broad host ranges will tend to be less speciose, so long as dispersal is high and barriers to gene flow remain relatively inconsequential. Nematodes that have narrow host ranges and lower virulence should in general be more species rich. The prediction that an increase in host range is concomitant with increased gene flow is likely countered by the rate of mortality of infected hosts (low mobility) and the insularity of the soil environment (Blouin *et al*., 1999). The effect (evolution of increasing virulence) is crudely reflected across larger phylogenetic space in Figure 243. That virulence could

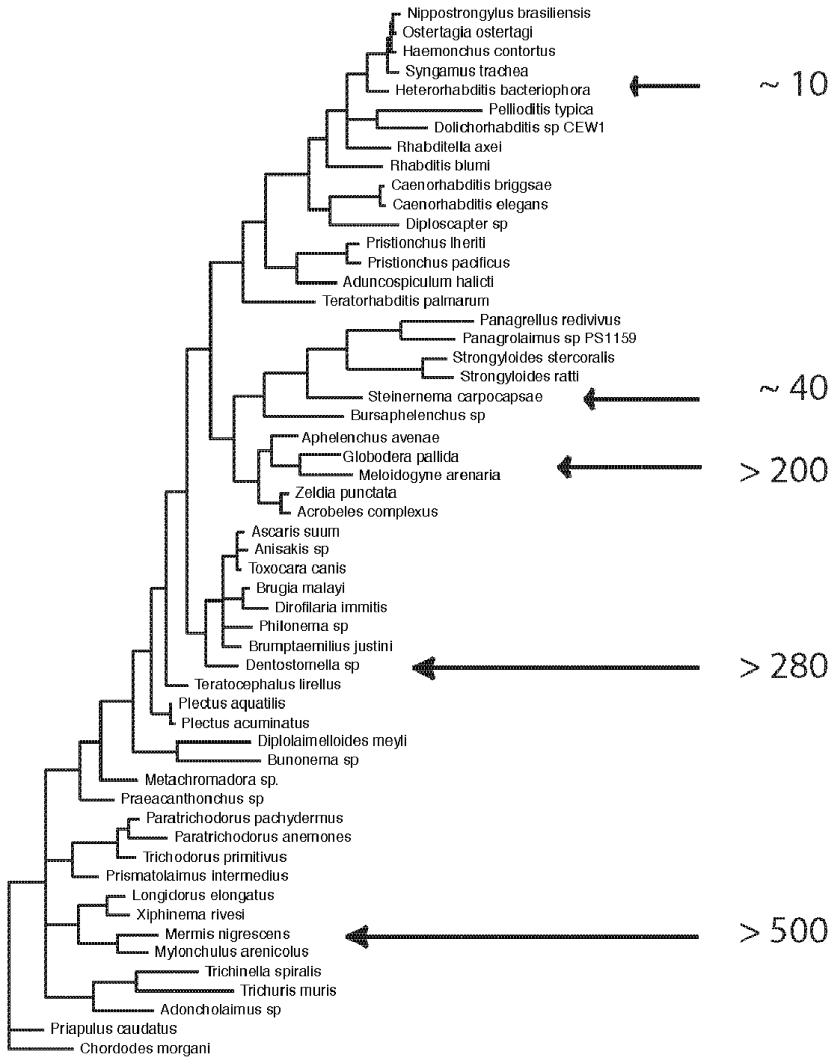

Fig. 243. *Species richness of select entomogenous nematode clades.* Heterorhabditis *and* Steinernema *have relatively few species, broad geographic ranges, little host fidelity and high virulence. Although it is predicted that an increase in host range is concomitant with increased gene flow, this is likely countered by the rate of mortality of infected hosts (low mobility) and the insularity of the soil environment (Blouin* et al.*, 1999). Note also the influence of sampling effort (some groups have been investigated more intensively than others) and species richness increases over evolutionary time (earlier branching taxa are also more speciose). Tree modified from Blaxter* et al. *(1998).*

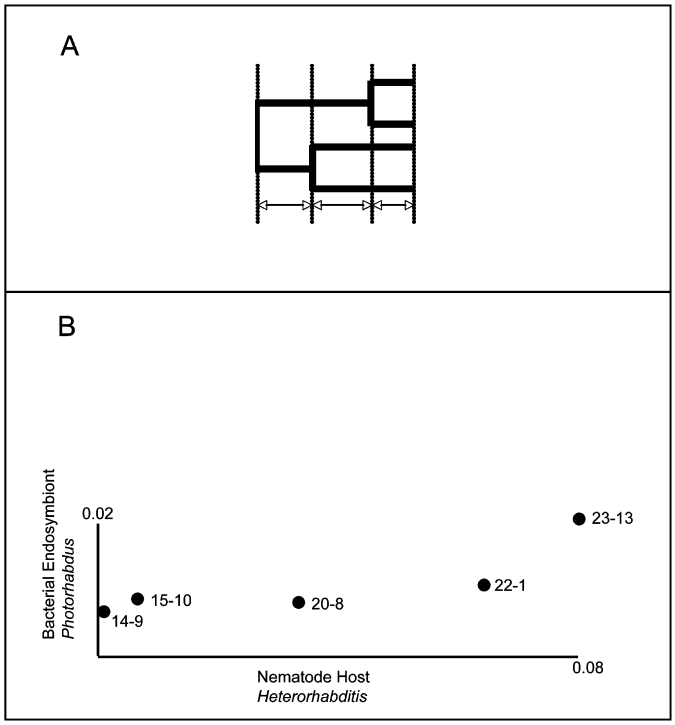

Fig. 244. *Plot of times to coalescence in units of genetic distance for each pair of* Heterorhabditis-Photorhabdus *cospeciation events. The coalescence time is the time two lineages last had a common ancestor. On an ultrametric tree this corresponds to the distance between the ancestral node and any one of its descendants (5A). Given ultrametric* Heterorhabditis *and* Photorhabdus *trees (not shown), plots of the coalescence time of each pair of cospeciation nodes (5B) reveal if the times to coalescence are correlated (they are:* $r = 0.82$*;* $P < 0.05$*) and also whether the timing of the codivergence events are pre-emptive (plotted line crosses a positive y-axis;* Photorhabdus *diverges earlier), synchronous (line crosses at the origin; they diverge at the same time) or delayed (line crosses a negative y-axis;* Heterorhabditis *diverges earlier (Page & Hafner, 1996). The number pairs of the plots refer to the paired nodes of the* Heterorhabditis-Photorhabdus *trees, respectively. The positive slope in the figure suggests pre-emptive evolution of the bacterium followed by a tracking response of the nematode host.*

be maintained, and even increased over evolutionary time, counters the widely held view that virulence is largely only manifest in the initial stages of symbiosis, and that the pathogenicity of the parasite decreases

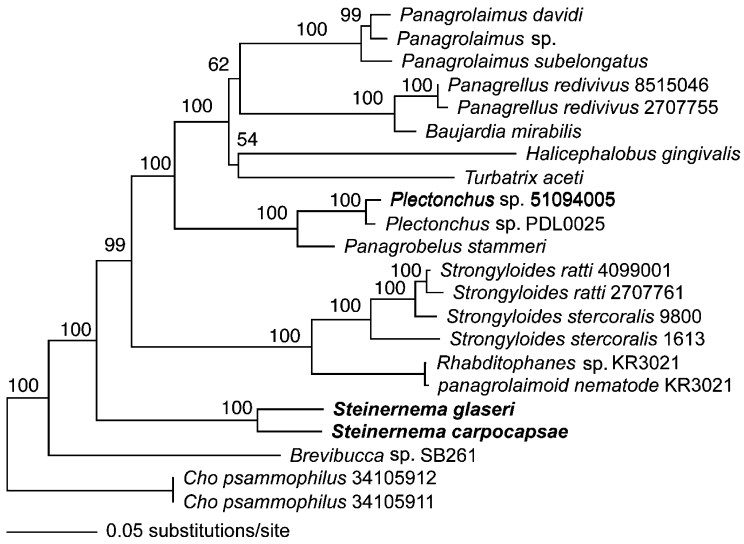

Fig. 245. *Phylogenetic position of* Steinernema *relative to 20 other most closely related nematodes for which comparable sequences are publicly available. Numbers on branches indicate node support (bootstrap, 1000 BioNJ reps) mapped on a tree produced with SSU sequences using the BioNJ neighbour-joining algorithm (Gascuel, 1997). Note sister taxon relationship with sampled Strongyloidoidea and Panagrolaimidae; see also Figure 243 and Nadler* et al. *(2006).*

over time. Work has shown that the evolution of virulence can be highly plastic and driven by adaptive virulence-associated responses by both host and parasite (Perlman & Jaenike, 2003), that often these responses can involve increases in virulence (Herre, 1993) and are correlated with a lack of concerted host-parasite coevolution (Perlman *et al*., 2003). In *Heterorhabditis* a piece of tenuous evidence supports the notion that the nematode is tracking changes that first occur in its bacterial symbiont. When times to coevolving coalescent events (time since two lineages shared a common ancestor)between species of *Heterorhabditis* and their bacterial endosymbionts *Photorhabdus* are plotted in terms of genetic distance, the correlation is significant ($r = 0.82$; $P < 0.05$) and the slope crosses the y axis positively, suggesting that the nematode is exhibiting a delayed response to evolutionary diversification of its symbiont even though the gene used to reconstruct the phylogenetic history of *Heterorhabditis* (ITS) is evolving at a faster rate than the gene used to reconstruct *Photorhabdus* phylogeny (16S) (Fig. 245).

Such analyses provide insight into the degree and mechanisms by which nematode and endosymbiont can influence each other's evolutionary trajectory. The small sample sizes and somewhat crude analyses caution that it is still preliminary to suggest that much, if anything, at this point can be inferred with confidence about correlations between the evolution of virulence, coevolution, host-range, gene flow and biodiversity, but EPN are well positioned to serve as model organisms for comparative and experimental research programmes in this area.

Phylogeny

Numerous molecular approaches have been applied to the evolutionary relationships of EPN. Initial efforts included PCR RFLP analyses of the internal transcribed spacer (ITS) regions of the ribosomal DNA tandem repeat unit (Reid, 1994; Reid *et al*., 1997). Other approaches included combinations of random amplified polymorphic DNA (RAPD) and morphological data (Liu & Berry, 1996). These papers still contain valid data, although their interpretations have been questioned due to analytical drawbacks, primarily insufficient numbers of phylogenetically informative characters, tenuous homology statements and tree building methods that are suboptimal for reconstructing evolutionary history (Stock *et al*., 2001). While the use of morphological characters in phylogenetic analyses remains robust, the performance of RAPD markers has fallen out of favour, primarily because of problems associated with replication and sensitivity of the approach.

The ribosomal small subunit (SSU, or 18S gene) was the first to be sequenced in an effort to resolve phylogenetic relationships within and between *Steinernema* and *Heterorhabditis* (Liu *et al*., 1997). While variation among species in both genera could be identified, the particular 5′ segment of the gene that was sequenced and analysed is evolving too slowly to produce the quantity of phylogenetically informative characters (substitutions) needed appropriately to infer relationships among species in their respective genera. Subsequently, the ribosomal large subunit (LSU), ITS and mitochondrial NADH dehydrogenase subunit 4 (ND4), and cytochrome oxidase I gene (Cox1) and small mitochondrial ribosomal RNA gene (16S) have been used to reconstruct the phylogenetic relationships among EPN (Adams *et al*., 1998; Liu *et al*., 1999; Szalanski *et al*., 2000; Stock *et al*., 2001; Nguyen *et al*.,

2001, 2004; Saeb *et al*., unpubl.). As the SSU is too conserved to be informative of relationships among most EPN species, the ITS, 16S, COI and ND4 appear to be evolving more rapidly than is optimal for phylogenetic resolution at this taxonomic level (Adams *et al*., 1998; Liu *et al*., 1999; Szalanski *et al*., 2000; Nguyen *et al*., 2001) where intraspecific and even intraindividual variability, although rare (see Adams *et al*., 1998; Spiridonov *et al*., 2004), exists. Their fairly rapid rate of evolution means that the ITS, 16S, COI and ND4 genes perform well at revealing relationships among closely related species, and even populations (Adams *et al*., 1998; Spiridonov *et al*., 2004) but they are unable to resolve more basal nodes with confidence (Adams, 2001; Nguyen *et al*., 2001; Spiridonov *et al*., 2004). Over its entire length, the LSU is highly conserved, but within it are stretches of expansion regions that have relatively higher substitution rates than the gene overall. The LSU (Duncan *et al*., 1999; Kaplan *et al*., 2000) D2D3 segment is one such expansion region that has been shown to evolve more slowly than the above mentioned genes but more rapidly than the SSU, making it (in most cases) more appropriate for resolving more deeply branching nodes in phylogenetic trees, while at the same time being sensitive enough to reveal differences among most species (De Ley *et al*., 1999; Duncan *et al*., 1999; Kaplan *et al*., 2000; Nadler *et al*., 2000; Tenente *et al*., 2004; He *et al*., 2005). This property also makes it, along with some of the more variable regions of the SSU, an appealing tool for species delimitation (Adams, 2001; Nadler, 2002; Sites & Marshall, 2004; Gozel *et al*., 2006b) and for use as a molecular barcode (Powers, 2004; Blaxter *et al*., 2005).

Whilst phylogenetic analyses of these genes appears to be straightforward, the ND4 and COI mitochondrial genes are protein coding genes that undergo selection to maintain an open reading frame, whereas the ribosomal genes are non-protein coding, and are thought to be under less selective constraint to maintain their length (Hillis & Dixon, 1991) (but note that there is some apparent selection pressure to maintain certain sequence and size fidelity for the proper maturation of the complete subunit (Van Nues *et al*., 1994)). Therefore, while insertion-deletion events (indels) are infrequent in the protein-coding genes, they are often the most common form of substitution in the ITS regions of EPN (Adams *et al*., 1998; Nguyen *et al*., 2001). Although robust models of nucleotide substitution have been developed for protein coding genes that incorporate different substitution parameters as a function of codon position, similar

models for rDNA (*i.e.*, that can account for indels and differential rates of substitution in stems *vs* loops of the RNA secondary structure) await full development. This is a problem for parsimony and model-based analyses. At the moment, the stem-loop issue can be addressed by determining the empirical transition/transversion ratios in stem and loop partitioned datasets and then analysing them under mixed model assumptions (Ronquist & Huelsenbeck, 2003) but see also (He *et al*., 2005). Indels can be accounted for by gap coding (Adams *et al*., 1998; Muller, 2006).

Optimal analyses of EPN phylogenetic relationships will incorporate every type of character for which homologous states can be identified across as many taxa as possible (Kluge, 1998, 2004; Giribet *et al*., 2001). However, the analyses of Liu and Berry (1996), Stock *et al*. (2001) and Spiridonov *et al*. (2004) remain the most deliberate attempts to integrate morphological and molecular characters in a cohesive analysis. As with all phylogenetic analyses, ongoing taxonomic work renders every previous solution obsolete as soon as it is published. Below are analyses that sample the taxa and comparable DNA sequence data currently available in public databases.

PHYLOGENY OF *STEINERNEMA* SPECIES

Spiridonov and Belostotskaya (1983) suggested that morphological features of *Steinernema* imply a most recent common ancestry with strongyloidids and alloionematids. Poinar (1993) subsequently inferred from morphological and bionomic characters that the Steinernematidae might have evolved from a proto-*Rhabditonema*-like ancestor. Subsequent analyses of SSU rDNA depict the Steinernematidae sharing common ancestry, if not sister taxon to the Strongyloidoidea and Panagrolaimidae (Blaxter *et al*., 1998, 2000) (Figs 243, 245).

Phylogenetic relationships among species of *Steinernema* have been carried out using morphological characters and RAPDs (Liu & Berry, 1996), PCR-RFLP of the ITS region (Reid, 1994; Reid *et al.*, 1997), and DNA sequences of the SSU (Liu *et al*., 1997), ITS, COII and 16S (Szalanski *et al*., 2000), ITS (Nguyen *et al*., 2001; Spiridonov *et al*., 2004) and LSU (Stock *et al*., 2001) genes. In the present analysis, we conservatively reconstruct phylogenetic relationships among nominal taxa using LSU sequences available in public databases (Fig. 246). Using the profile alignment mode of Clustal X (Thompson *et al*., 1997), individual sequences were aligned to the most complete alignment of

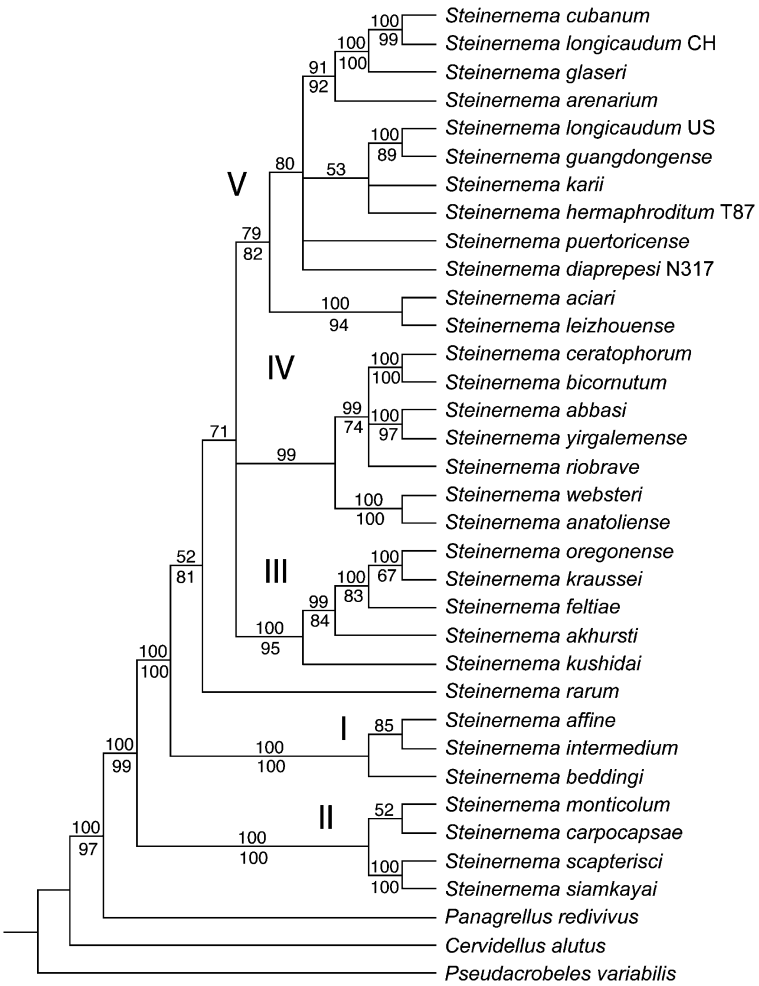

Fig. 246. *Phylogenetic relationships among species of* Steinernema *based on LSU rDNA sequence data, summarising Bayesian, likelihood and parsimony analyses. Values above each branch represent Bayesian posterior probabilities, parsimony bootstrap indices (1000 replicates), where concordant with the Bayesian analyses, appear below. Clade designations (I-V) follow Spiridonov* et al. *(2004).*

Stock *et al*. (2001) available in TreeBase (Sanderson *et al*., 1994). For the parsimony analyses all characters were considered unordered and unweighted. Of 1159 total aligned characters, 538 are constant and 438 are phylogenetically informative. One hundred eighty-three characters

varied but are likely phylogenetically uninformative autapomorphies. Gaps were treated as missing data. Starting trees for the heuristic search were obtained by stepwise addition with a random addition sequence, with TBR branch-swapping. Parsimony bootstrapping was carried out the same way, but with 1000 replicates.

Maximum likelihood searches were carried out based on the GTR + G + I model of nucleotide evolution (Posada & Crandall, 1998) with six substitution types and substitution rate matrix and nucleotide frequencies estimated from the empirical data (transformation series). The model assumed that approximately 15% of the sites (homologous nucleotides) were invariable, with the variable sites approximating a gamma distribution (shape parameter 0.6213, four rate categories). Heuristic searches were carried out the same way as described above for the parsimony analyses.

Bayesian analyses were performed using MrBayes 3.1 (Huelsenbeck & Ronquist, 2001) based on the same models used for the ML searches. For each analysis we carried out runs for 7 000 000 generations with four incrementally heated chains and then sampled at intervals of 1000 generations to include 7000 data points. Stationarity was estimated by plotting (with Microsoft Excel 2004) the log likelihood scores against generation time and assumed stationarity when the curves flattened out. This phase was reached between 5000 and 6000. The first three to six trees (burn-in) were discarded and 50% majority rule trees were obtained from the remaining 6994-6995 data points with the purpose of obtaining the posterior probability values. To avoid local entrapment on a suboptimal peak in the tree space, we performed two independent analyses and compared these for convergence to similar log likelihood mean values (Huelsenbeck & Bollback, 2001; Leache & Reeder, 2002). We also compared the posterior probabilities for individual clades obtained from the separate analyses for congruence to ensure convergence of the two analyses. All phylogenetic analyses were performed on a RackSaver 64 node dual Opteron processor computing cluster. The strength of support for nodes in the Bayesian tree is estimated by posterior probabilities. For Bayesian analyses, posterior probabilities greater than 0.95 are typically considered robust (Leache & Reeder, 2002), 0.70 for maximum parsimony and maximum likelihood bootstrap analyses (Hillis & Bull, 1993).

The phylogeny presented in Figure 246 presents few deviations from previous hypotheses (Nguyen *et al*., 2001; Stock *et al*., 2001; Spiridonov

et al., 2004) but includes several taxa previously unrepresented in comprehensive phylogenies of the genus, namely: *S. guangdongense*, *S. hermaphroditum*, *S. aciari*, *S. leizhouense*, *S. yirgalemense*, *S. websteri*, *S. anatoliense*, *S. akhursti* and *S. beddingi*. *Steinernema pakistanense*, *S. weiseri*, *S. tami* and *S. neocurtillae* are sampled in the analyses of Spiridonov *et al*. (2004) but are not included in the present analysis for lack of LSU sequence data for these taxa. *Steinernema pakistanense* most likely nests within clade IV, *S. weiseri* within clade III and *S. tami* within clade II. The position of *S. neocurtillae* within the Steinernematidae can be inferred to diverge somewhere between clades I and II, but its placement is one of the most enigmatic (in terms of support) of all the taxa in the genus (Nguyen *et al*., 2001; Spiridonov *et al*., 2004). Another problematic taxon is *S. rarum* (see discussion below). The addition of taxa to a dataset is typically seen as more important than adding more characters, particularly where there may be problems of long-branch attraction (LBA) (Graybeal, 1998; Poe, 1998). However, the only potential LBA event in *Steinernema* involves the branch between the species in clade II and the rest of the genus, and none of the additional taxa appears to intersect this branch.

The overall structure of the *Steinernema* tree is not completely resolved without some ambiguity; clades II, IV and V all contain taxa with weakly supported relationships, and *S. rarum* appears not to belong to any predetermined clade at all. Spiridonov *et al.* (2004) placed this taxon as sister to the remaining members of clade V, Stock *et al*. (2001) depict this taxon as sister to clades III and IV, and the present analyses suggest it is more likely the sister taxon to clades III, IV and V.

One of the earliest morphological trends to emerge from mapping character traits on phylogenetic trees was the length of the infective juveniles where there appeared to be clades of 'long' and 'short' morphs (Reid, 1994; Reid *et al*., 1997) but, as analyses have since become inclusive of increasing numbers of taxa, this character has been shown to be fairly plastic and homoplasious (Stock *et al*., 2001). Although Stock *et al*. (2001) suggest that the majority of morphological characters commonly used for classification and phylogenetic relationships are either homoplastic or plesiomorphic, Spiridonov *et al*. (2004) suggested that the structure of bacterial vesicles, colour of male copulatory structures, position of the excretory pore and sperm morphology could be useful synapomorphies for future phylogenetic analyses. Detailed information on steinernematid morphological characters is provided elsewhere (see

Chapter 3), but in the context of the present phylogeny it would be interesting to see if, for example, *S. beddingi* has yellow to colourless spicules and whether *S. guangdongense* and *S. hermaphroditum* lack a true bacterial vesicle as predicted by Spiridonov *et al*. (2004). It is also possible that *S. websteri* and *S. anatoliense* actually have horn-like cephalic structures (in contradiction to their original descriptions), although this observation requires examination of exsheathed infective juveniles as it is easily overlooked (Nguyen & Adams, 2003).

PHYLOGENY OF *HETERORHABDITIS* SPECIES

Poinar (1993) suggested that, based on morphological, physiological, distributional and biological evidence, *Heterorhabditis* most likely shared a most recent common ancestor with a *Pellioditis*-like ancestor. This argument is further corroborated by SSU gene genealogy (Fig. 247).

Virtually all molecular systematic approaches to relationships among species and populations of *Heterorhabditis* have been done using the ITS rDNA gene (Adams *et al*., 1998, 2006; Phan *et al*., 2003; Nguyen *et al*., 2004, 2006), although the ND4 gene has also been used effectively (Blouin *et al*., 1999; Liu *et al*., 1999). In the present analysis, we conservatively reconstruct phylogenetic relationships among nominal taxa using ITS and ND4 sequences available in public databases. Accordingly, individual ITS sequences were aligned using the profile alignment mode of Clustal X to the most complete alignment of Adams *et al*. (1998). Alignment of the ND4 sequences was straightforward as there were no insertion or deletion events.

For the parsimony analyses of the ITS sequences, all characters were considered unordered and unweighted. Of 1167 total aligned characters, 533 were constant and 389 were phylogenetically informative. Some 243 characters varied, but were probably phylogenetically uninformative autapomorphies. Gaps were treated as missing data. Starting trees for the heuristic search were obtained by stepwise addition with a random addition sequence with TBR branch swapping. Parsimony bootstrapping was carried out the same way, but with 1000 replicates. Likelihood analyses were run using the GTR-G model selected by AIC (Posada & Crandall, 1998; Posada & Buckley, 2004) (six substitution types, empirical rate matrix and nucleotide frequencies with no invariable sites and a gamma distribution of rate shape parameter of 0.9 across four

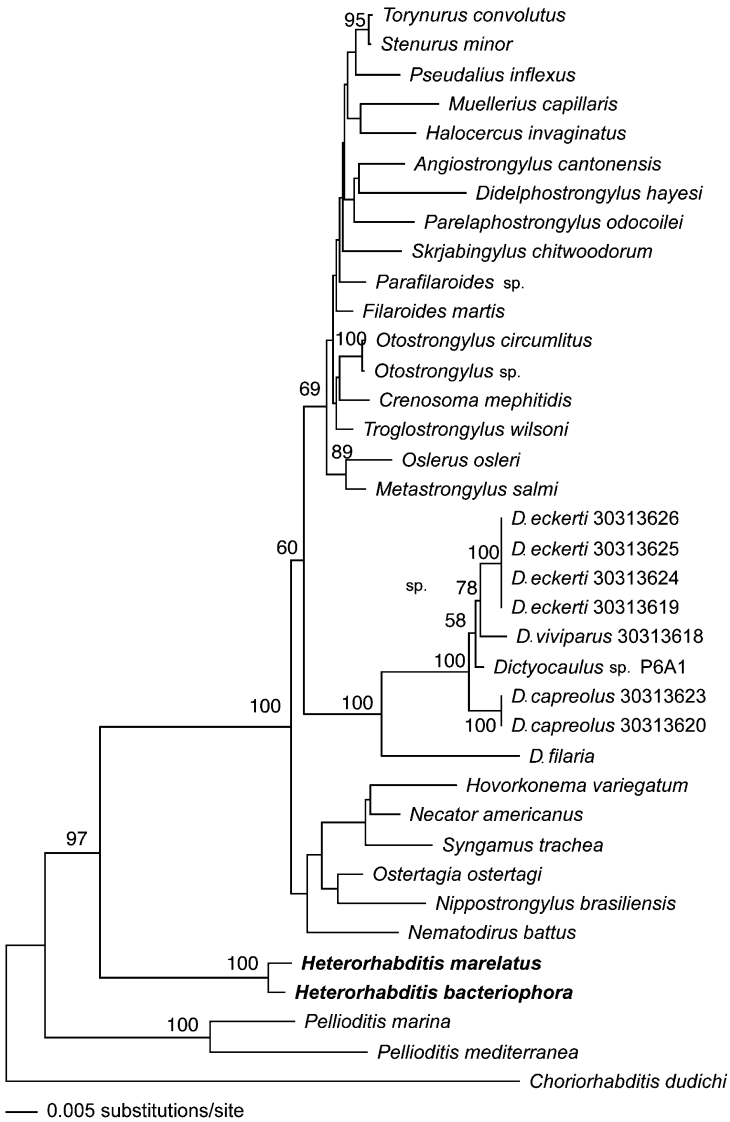

Fig. 247. *Phylogenetic position of* Heterorhabditis *relative to 35 other most closely related nematodes for which comparable sequence information is publicly available. Numbers on branches indicate node support (bootstrap, 1000 reps) mapped on a tree produced with SSU sequences using the BioNJ neighbour-joining algorithm (Gascuel, 1997). Note sister taxon relationship to the Filaroides and Strongyloidea; see also Figure 243 and Hoglund* et al. *(2003).*

rate categories). Starting trees were obtained by stepwise addition with random addition sequence and TBR branch-swapping.

For the parsimony analyses of the ND4 sequences, of 495 total characters 173 were informative; 257 were constant, and 65 varied but were parsimony-uninformative. Tree search strategy was the same as that described above for the ITS analyses. Likelihood analyses of the ND4 data matrix assumed a GTR + I + G model of sequence evolution, as estimated by ModelTest (Posada & Crandall, 1998). Accordingly, six substitution types, their rate matrix and nucleotide usage frequencies were estimated empirically from the multiple sequence alignment. Approximately 4% of the inferred homologous sites were considered to be invariant, with the distribution of rates at the variable sites approximating a gamma distribution with a shape parameter of 0.36 and four rate categories (mean rate for each category). Bayesian analyses for the ITS and ND4 regions employed the same models of sequence evolution as were utilised in the ML analyses and proceeded as described above for the *Steinernema* LSU analyses.

There is general topological agreement between the two trees except for the placement of *H. zealandica* and *H. marelatus* to sister taxa *H. downesi* and *H. megidis* (Figs 248, 249). The ITS solution depicts *H. marelatus* as sister to *H. downesi* + *H. megidis*, whereas the ND4 solution places *H. zealandica* as sister taxon to this clade. The node associated with this discrepancy is tenuously supported by Bayesian posterior probability and parsimony bootstrapping and is consistent with previous analyses (Adams & Nguyen, 2002), which also document the discordance between the present ND4 tree topology and that of Liu *et al*. (1999).

Species delimitation

Delimitation of nematode species

The body of literature surrounding species and species concepts is amongst the largest in biology. Much progress has been made, both in terms of determining what species are (ontology) and how best to find them (epistemology) but, upon inspection, the majority of metazoan species descriptions published over the last 10 years do not readily reflect the progressive trend. This is not surprising. The language of the best ideas that this literature has to offer is dense and burdensome to read,

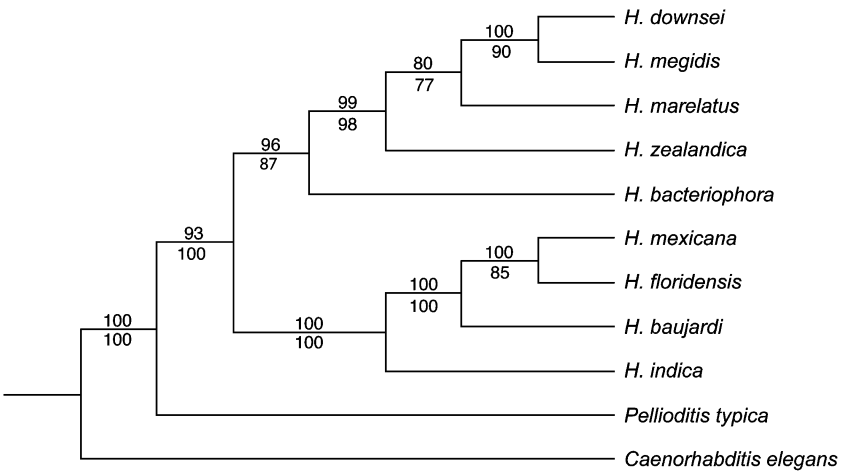

Fig. 248. *Phylogenetic relationships among species of* Heterorhabditis *based on ITS sequences, summarising Bayesian, maximum likelihood and parsimony analyses. Values above each branch represent Bayesian posterior probabilities. Parsimony bootstrap indices (1000 replicates) appear below (where trees are concordant).*

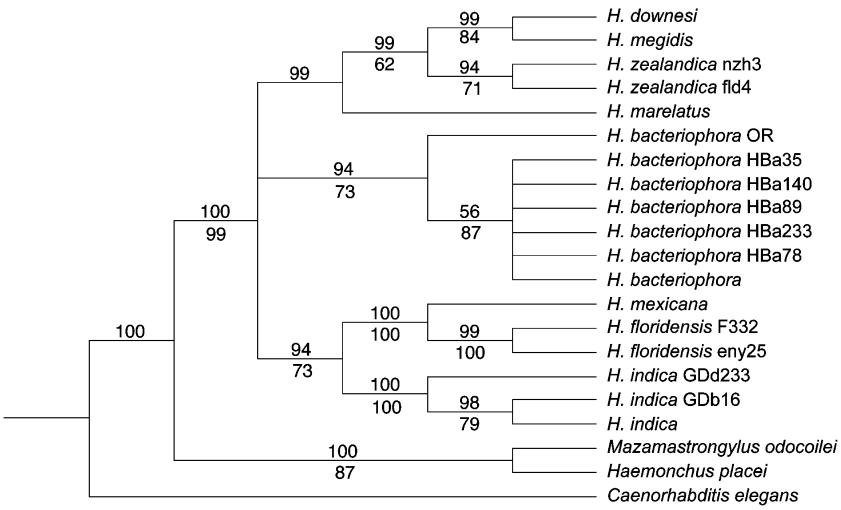

Fig. 249. *Phylogenetic relationships among species and some populations of* Heterorhabditis *based on ND4 mtDNA sequence data, summarising Bayesian, maximum likelihood and parsimony analyses. Values above each branch represent Bayesian posterior probabilities, parsimony bootstrap indices (100 replicates), where concordant, appear below.*

if not outright impenetrable – for example, a sure cure for insomnia is Adams (1998). The result is that, despite the philosophical and analytical tools that have been developed to recover and represent evolutionary history accurately, few taxonomists are actually taking advantage of them. Few species descriptions are explicit as to what notion of species they are attempting to delimit, or the analytical tools they are using to do so. The confusion surrounding definitions of species is not unlike the definition of what is 'obscene' under United States law. In 1964, Justice Potter Steward summarised the court's position as indefinable, but that "I know it when I see it . . ." (Anon., 1964). As applied to taxonomy, this problem defers to the common quip that a species is "what a good taxonomist says it is" (Crum, 1985). Not state of the art, but *status quo* nonetheless. Thus it appears "[taxonomists] defend the *status quo* long past the time when the *quo* has lost its status" (with apologies to Peter; Peter & Hull, 1969).

Analytical methods for morphological and molecular data types vary somewhat, but there is no conceptual difference for their use in systematics and conflicts between morphological and molecular approaches have been overemphasised. The divisions that do exist are being bridged rapidly by research in developmental and molecular genetic processes and systematic theory. Both types of data can be used to identify, diagnose and delimit species. As with all scientific endeavours, systematic hypotheses (including the validity of species and their phylogenetic relationships) are supported or rejected based on the preponderance of evidence. The most robust hypotheses are those that withstand falsification by the greatest amount of corroborative evidence – whether molecular, morphological or behavioural. The only prerequisite for systematic data (characters) is that they have a heritable genetic basis, and demonstrate the level of fixation and variation appropriate to resolve the question at hand.

The identification and diagnosis of species is often confused with species delimitation, or the process of testing the hypothesis that the entity in question *is* a species. Identifying species involves the recognition of differences and similarities among described taxa. Diagnosis is merely the description of the taxon. Less intuitively, species delimitation involves making a determination and prediction about the history and fate of an evolutionary lineage. The theoretical difference between delimitation and diagnosis is that the former results in something that has meaning in a historical, evolutionary context. A diagnosis cannot make

the same claim. In practice, species delimitation differs from diagnosis in that it requires character argumentation, or determining whether homologous characters are plesiomorphic (primitive) or apomorphic (derived). In contrast, diagnosis consists of simply determining whether homologous characters are 'similar' or 'different'. It is crucial that systematists make this distinction because the two activities are as disparate as saying, "this entity looks different" and "this entity is a species" – see Kiontke *et al*. (2002) and Sudhaus and Koch (2004) for clear examples of this exercise.

The most salient theoretical definition of species is the evolutionary species concept. According to this concept, a species is "an entity composed of organisms that maintains its identity from other such entities through time and over space, and which has its own independent evolutionary fate and historical tendencies" (Wiley & Mayden, 2000). The best way to find species is to identify independent evolutionary lineages. There are numerous ways to achieve this, depending on the biological properties of the nematodes, and the types of data that are accessible. The particular mechanics of these methods are described as those that emphasise the tokogeny-phylogeny interface (*i.e*., incorporate inferences about gene flow) (Sites & Marshall, 2003). The gist of these approaches is that if a lineage (species) is evolving independently, it will accumulate fixed changes among its populations that do not appear in any other lineage. Conversely, shared or variable character states are indicative of non-exclusive reticulation and reflect genealogical patterns consistent with outcrossing populations. A cartoon illustrating these principles is presented in Figure 250.

Delimitation of EPN species

Although the first EPN species was described in 1923, over 80% of them have been described since 1990 (Adams *et al*., 2006). The recent increase in species descriptions is driven primarily by the potential for these species to find applications in biological control, but they are quickly becoming the focus of basic biological questions of coevolution (ffrench-Constant *et al*., 2003; Ciche *et al*., 2006) and ecology (Lewis *et al*., 2006), which also spurs the search for biodiversity.

Thus far, virtually all species of entomopathogenic nematodes have been delimited in Linnaean (phenetic) fashion (Mayr, 1963) based on diagnosable differences in morphology, morphometrics, and even mole-

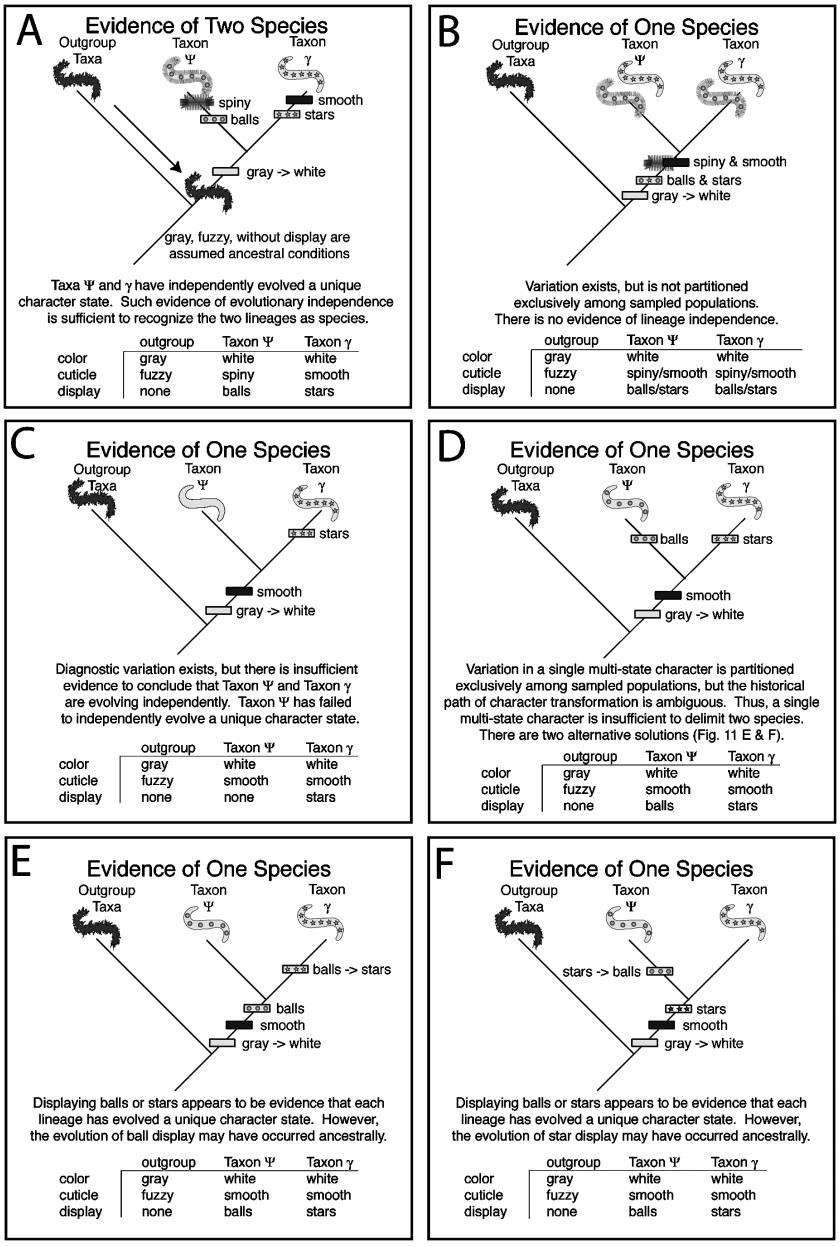

Fig. 250. *Examples of discovery operations required to delimit species. Character states are for illustrative purposes only and are not meant to depict those actually found in entomopathogenic nematodes.*

cules. In some cases crossbreeding tests are done to examine potential reproductive isolation to clarify whether the criteria of biological species can be met (Mayr, 1964). However, despite the thoroughness of these descriptive investigations, delimiting species solely on the basis of overall similarity or potential to interbreed can misrepresent the actual number of unique evolutionary species that exist in nature and their inferred historical relationships. For example, delimitation in the same group based on reproductive compatibility can result in a situation where two sister populations can be reproductively isolated, but each is interfertile with a more distantly related population (Adams *et al*., 1998). Delimitation based on overall similarity or reproductive compatibility betrays historical relationships and probably fails to accurately reflect the number of species that actually exist (Adams, 1998).

Adams (1998) argued that lineages that evolve unique character states show evidence of lineage independence. Identifying unique characters (autapomorphies) requires character argumentation (polarisation) *via* outgroup comparison with all the other known members of the group. The requirement that species possess autapomorphies protects against misrepresenting the actual number of species and ensures that all valid species are consistent with evolutionary history (Adams, 1998). Spiridonov *et al*. (2004) take exception to this point. As evidence, they point to instances where their molecule of choice (ITS) failed to yield autapomorphies for either of two sister taxa (in this case *S. karii* and *S. siamkayai*). This leads to their conclusion that "autapomorphies for some well established species may disappear as the number of species studied increase" and that therefore the utility of autapomorphies for species delimitation is not a "sound procedure" (see Spiridonov *et al*., 2004, pp. 560-561). We disagree that sampling additional

Fig. 250. *(Continued). A: Demonstration of lineage exclusivity (independent evolution of autapomorphic character states), evidence of two species; B: Sampled populations are polymorphic, no evidence of lineage independence; C: Diagnostic variation exists in the form of a unique combination of characters, but taxon* Ψ *does not show evidence of independent evolution (is a privative group). D-F: The reason a single multi-state character cannot be used to delimit two species. D: Character states for a fixed, multi-state character appear to be partitioned exclusively among taxa* Ψ *and* γ*, but there are two equally logical paths (E, F) that are operationally indistinguishable from the scenario presented in C.*

loci from additional populations and analysing them with an eye for autapomorphies implies that Adams' approach is flawed or will result in decreased systematic accuracy. Nor are we troubled by the fact that additional data might suggest that what we think are two 'well established' species in reality represent two or more populations of a single species. Where morphological data suggest otherwise, the addition of molecular characters usually supports the original species descriptions (Nadler *et al*., 2000) or sheds light on cryptic speciation (Gozel *et al*., 2006a). Also, if the two species are indeed well supported by other characters (autapomorphies?), then the absence of autapomorphies in the ITS data is irrelevant because there is no *a priori* reason to believe that the molecular characters are somehow superior to the morphological characters used in the original or subsequent species descriptions. As an alternative to phylogenetic methods for delimiting species, Spiridonov *et al*. (2004) suggest that sequence divergence is a better indication of lineage independence. Unfortunately, sequence divergence cannot reveal lineage independence and, therefore, is a suboptimal tool to find independent lineages (Farris, 1980, 1981, 1983). The use of sequence divergence alone to delimit species is arbitrary; no more informative of lineage divergence than say, body length divergence. It is a poor indicator of species boundaries (Ferguson, 2002). We caution those who wish to interpret them in this manner.

Molecular methods

The allure of molecular data for work in systematics is their clear genetic basis, a compelling advantage given the significant amount of environmental and host-induced morphological variation displayed by entomopathogenic nematodes (Nguyen & Smart, 1996; Hominick *et al*., 1997). Potentially sensitive, objective and powerful, molecular characters can also be misleading when wielded carelessly. The use of common genetic markers for EPN research, together with a discussion of the relative merits of using DNA sequencing methods as opposed to RAPD, PCR-RFLP and AFLP markers, has been addressed previously (Adams & Nguyen, 2002; Adams *et al*., 2006). Below we provide some basic protocols for acquiring DNA sequence data for use in the molecular systematics of EPN.

DNA EXTRACTION

DNA extraction from individual nematodes

For most applications, it is important that DNA template used for RFLP or DNA sequencing come from individual nematodes because variation within and between individual animals must be accounted for. This is especially important when working with variable markers that are informative at the species boundary. Individuals of any sex or stage may be used, but large females commonly yield the largest amounts of DNA.

DNAzol® DNA extraction (modified from Steve Nadler, pers. comm.)

Place a single nematode in a 0.2 ml PCR tube with no more than 5 μl of water. Add 100 μl of the digestion solution to the tube and incubate overnight at 56°C, vortexing the tube occasionally. Check tissues for digestion using a dissection or other low-powered light microscope. If undigested, add 1 μl proteinase K and incubate longer (eggs will not usually digest). 'Pump-mix' digested tissues. Centrifuge the sample for 5 min at 10 000 *g* in a microfuge.

Use a pipette to remove *ca* 90 μl of the supernatant and place it in a new 0.2 ml PCR tube (the important thing is to leave the last *ca* 10 μl containing the cellular debris at the bottom of the tube to be discarded). Heat kill the proteinase by incubating the tube at 95°C for 15 min in a thermal cycler with heated lid (hot bonnet).

Add the contents of the PCR tube from step 4 to 250 μl of DNAzol® in a new 1.7 ml Eppendorf tube. Add 4 μl of the Polyacryl carrier (stored in refrigerator at 4°C; mix the carrier well before using).

Mix the contents of the tube by inverting it *ca* five times. Add 250 μl of 100% ethanol. Mix by inversion ten times. Let sample sit at room temperature for 5 min.

Pellet DNA by centrifugation at 5000 *g* for 5 min at room temperature (spin with cap 'tabs' facing out).

Wash the DNA twice with 800 μl of 75% ethanol. To wash, invert the tube containing the sample and ethanol *ca* six times. Briefly centrifuge the tube to pellet the DNA/carrier pellet, if necessary (if it has broken loose). Carefully decant the ethanol (or remove by pipetting as required).

Remove remaining alcohol from the tube using a fine pipette tip/pipettor. Do NOT spin-dry the DNA/carrier. Once excess ethanol is removed, leave tubes open in the refrigerator (ensure that tubes are slightly covered or tilted on their side to prevent contamination) or on

ice in a hood for 1-2 h to allow DNA pellet to dry. This should be done in the refrigerator or on ice under a hood. Once all ethanol has evaporated and the pellet has dried, resuspend the DNA pellet in 6-10 μl of TE. Try using 2 μl of the resuspension for a 25 μl PCR reaction, though more can be used if the DNA concentration is low.

- Preparation 100 mM Tris-HCl pH 7.6
  Tris hydroxymethyl 15.8 g
  Distilled water to 1000 ml

Dissolve 15.8 g of Tris in 800 ml of distilled water. Adjust pH with NaOH. Add distilled water to bring the solution to 1000 ml. Sterilise by autoclaving at 121°C for 15 min. Store at room temperature.

- Preparation 200 mM NaCl
  Sodium chloride 11.7 g
  Distilled water to 1000 ml

Dissolve 11.7 g of NaCl in 800 ml of distilled water, and then add distilled water to bring the solution to 1000 ml. Sterilise by autoclaving at 121°C for 15 min. Store at room temperature.

- Preparation 0.5 M EDTA pH 8.0
  EDTA 186.1 g
  Distilled water to 1000 ml

Add 186.1 g of EDTA in 500 ml of distilled water stir and heat at 30°C. Add *ca* 100 ml of 1 M NaOH to bring the pH to 8.0. Add distilled water to bring the solution to 1000 ml while adding 1 M NaOH as needed for pH. Sterilise by autoclaving at 121°C for 15 min. Store at room temperature.

- Preparation 1 M NaOH (for making EDTA)
  Sodium hydroxide 40 g
  Distilled water to 1000 ml

Dissolve 40 g of sodium hydroxide in 800 ml of distilled water, and then add distilled water to bring the solution to 1000 ml. Sterilise by autoclaving at 121°C for 15 min. Store at room temperature.

- Preparation 10% Sarkosyl
  Sodium N-lauroyl sarcosine 100 g
  Distilled water to 1000 ml

Dissolve 100 g of sodium N-lauroyl sarcosine in 800 ml of distilled water, then add distilled water to bring the solution to 1000 ml. Sterilise by autoclaving at 121°C for 15 min. Store at room temperature.

- Preparation Proteinase K

Proteinase K	0.1 g
Distilled water	to 10 ml

Dissolve 0.1 g of proteinase K in 9 ml of distilled water, then add distilled water to bring the solution to 10 ml. Aliquot this into 0.2 PCR tubes and store at −20°C.

- Preparation Digestion Solution

100 mM Tris HCl pH 7.6	200 μl
200 mM NaCl	200 μl
10% Sarkosyl	200 μl
0.5 M EDTA pH 8.0	400 μl
Proteinase K (10 mg ml^{-1})	20 μl
Ultrapure water	980 μl

(Final solution is 10 mM Tris-HCl pH 7.6, 20 mM NaCl, 100 mM EDTA pH 8.0, 1% Sarkosyl, 0.1 mg ml^{-1} proteinase K.)

Worm Lysis Buffer DNA extraction (modified after Jones et al.*, 2006)*

This method is rapid and yields the largest volumes of template for PCR, but does not work equally well with all taxa (works poorly for some Tylenchomorpha). Place a single nematode in a 0.2 ml PCR tube with no more than 5 μl of water. Add 40 μl of the digestion solution to the tube and incubate at 60°C for 1 h or until the individual is completely digested. Vortex the tube occasionally. Manually rupturing the individual organism can aid in rapid and complete digestion. This can be done by using a pipette tip or pulled Pasteur pipette with a blunted end.

Check tissues for digestion using a dissection or light microscope. If undigested, add 1 μl proteinase K and incubate longer (eggs will not usually digest). 'Pump-mix' digested tissues.

Heat-kill the proteinase by incubating the tube at 95°C for 15 min in a thermal cycler with heated lid (hot bonnet). Use 2-5 μl of lysate as the DNA template in a 25 μl or 50 μl PCR reaction. Lysate should be stored at −20°C or −80°C.

- Preparation 100 mM Tris pH 8.2

Tris (hydroxymethyl) aminomethane	12.1 g
Distilled water	to 1000 ml

Dissolve 12.1 g of Tris in 800 ml of distilled water. Adjust pH with NaOH. Add distilled water to bring the solution to 1000 ml. Sterilise by autoclaving at 121°C for 15 min. Store at room temperature.

- Preparation Worm Lysis Buffer 10 ml

Proteinase K	10 mg
100 mM Tris pH 8.2	1 ml
KCl	37.3 mg
$MgCl_2$	5.1 mg
Tween-20	45 μl
NP-40 (IGEPAL)	45 μl
Gelatin	5.0 mg
Ultrapure water	to 10 ml

(Final solution is 1 mg ml^{-1} proteinase K, 10 mM Tris pH 8.2, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% Tween-20, 0.45% NP-40, 0.05% gelatin.)

Dissolve 10 mg of proteinase K, 37.3 mg of KCl, 5.1 mg of $MgCl_2$, and 5.0 mg of gelatin in 8 ml of ultrapure water. Then add 1 ml of 100 mM Tris (pH 8.2), 45 μl of Tween-20, and 45 μl of NP-40. Add ultrapure water to bring the solution to 10 ml. Store at 4°C in the refrigerator.

DNeasy® DNA extraction

Another extraction method that is effective in isolating nematode DNA is the Qiagen DNeasy® tissue kit, which is our method of choice for ethanol preserved specimens. Use the protocol for purification of total DNA from animal tissues with the following modifications: In step 1, a single nematode is manually crushed using a pipette tip or pulled Pasteur pipette with a blunted end. Follow step 2 but incubate at 60°C for 2-3 h while vortexing every 15 min. Follow the rest of the steps as written in the DNeasy® tissue handbook. After the final step, dry the sample down using a vacuum speed dryer for 2 h or until dry and resuspend the DNA in 20 μl of ultrapure water or TE.

SEQUENCE ANALYSIS

Polymerase chain reaction (PCR)

PCR is a powerful molecular technique used to amplify target genes, although the specifics vary depending on the primers and commercial DNA polymerase that is used. Here is a generic PCR protocol that works with many brands of Taq and several primer sets as well. All reagents should be stored at −20°C or −80°C. Thaw reagents on ice:

Buffer (commercially available Taq comes with its own specific buffer)
Thermus aquaticus (Taq) DNA Polymerase

DNA Primers (both forward and reverse)
Deoxynucleotide triphosphates (dNTPs)
Ultrapure water
DNA template (from extraction or other source)

Once the reagents have completely thawed, they are combined in a 1.5 ml Eppendorf tube in the following order and quantities, forming the reaction mixture also known as the master mix. This step avoids pipetting minute amounts into multiple individual tubes:

Reagent	1 Reaction	10 Reactions	x Reactions
ThermoPol buffer	2.5 μl	25 μl	$2.5 \times x$ μl
Ultrapure water	17.3 μl	173 μl	$17.3 \times x$ μl
Forward Primer	1.25 μl	12.5 μl	$1.25 \times x$ μl
Reverse Primer	1.25 μl	12.5 μl	$1.25 \times x$ μl
dNTPs	0.5 μl	5 μl	$0.5 \times x$ μl
Taq	0.2 μl	2 μl	$0.2 \times x$ μl
Total Master Mix Volume	23 μl	230 μl	$23 \times x$ μl

To allow for pipetting error, it is recommended that one extra reaction be added to the calculation for every ten reactions needed. The master mix should be mixed by pump mixing, flicking the tube or gently inverting.

Transfer 23 μl of reaction mixture into a PCR tube for each DNA template.

Add 2 μl of DNA template to each PCR tube for a total reaction volume of 25 μl. If more DNA template is used, subtract the extra volume from the water added so that each reaction still has a total volume of 25 μl.

Load tubes into thermal cycler. The exact cycling parameters used depend on the gene targeted, or in other words the primers being used. The following cycling parameters are generic and work with a variety of primer sets, although optimisation may be required in order to obtain the best results for your primer set.

Steps	*Step instructions*
1. Initial denaturation	Incubate at 95°C for 10:00 min
2. Denaturation	Incubate at 94°C for 1:00 min
3. Annealing	Incubate at 50°C for 1:00 min

4. Elongation	Incubate at 72°C for 2:00 min
5. Cycling step	Cycle to step 2 for 39 more times
6. Final elongation	Incubate at 72°C for 10:00 min
7. Cycle termination	Incubate at 4°C forever

Once the thermal cycler reaches step 7, the cycle can be stopped and the tubes with PCR product removed. PCR product should be stored at −20°C or −80°C and should always be kept on ice when in use.

- Preparation Primer Resuspension

DNA forward and reverse primer	lyophilised
Ultrapure water	variable

Primers are shipped lyophilised. To make a master stock, check the number of nanomoles on the tube. Multiply this number by 10 then add that number of μl of ultrapure water to the tube. For example, in a tube with 72.5 nm of primers, add 617 μl of ultrapure water. This creates a final stock of about 100 pmol μl^{-1} which should be stored at −20°C or −80°C. Mix by inversion or by gently vortexing. Working stocks of primer for PCR should be 20 pmol μl^{-1} by diluting at a ratio of 1 : 5. This is done by adding 20 μl of your final stock primer to 80 μl of ultrapure water in a 0.2 ml PCR tube.

- Preparation dNTP solution

100 mM dATP	62.5 μl
100 mM dCTP	62.5 μl
100 mM dGTP	62.5 μl
100 mM dTTP	62.5 μl
Ultrapure water	2.25 ml

Pipette 2.25 ml of ultrapure water into a 15 ml conical tube, then pipette 62.5 μl of each dNTP solution into the tube. Mix by inversion or by gently vortexing. Aliquot 500 μl of the mixture in five separate 0.5 ml tubes to minimise freeze thawing and to prevent contamination. This solution should be stored at −20°C or −80°C and should always be thawed on ice before use.

Agarose gel electrophoresis

PCR reactions can be verified and quantified using gel electrophoresis. Make a 1% agarose gel by adding 0.5 g agarose to 50 ml of 1X TBE. This is heated on a Bunsen burner or in a microwave until the solution is uniform with no visible agar grains. While still warm but no longer steaming add 4 μl of ethidium bromide, which allows the DNA to be visualised

in the gel. Pour the still warm mixture into a gel mould. Allow the gel to cool, setting in combs that provide the amount of wells as needed.

Once solid, the gel can be transferred to a gel box and submerged in 1X TBE. PCR product, thawed on ice, can be added to the individual wells by pipetting 3 μl of DNA tracking dye onto a sheet of Parafilm® for each sample to be run. Then 5 μl of PCR product is added to the bead of tracking track, pump mixed, and then pipetted into a well. DNA ladder should be added in the same manner but using 1 μl of ladder (amount of ladder required will vary by manufacturer and concentration) and 1 μl of tracking dye. The purpose of the dye is to allow the otherwise clear PCR product to be visualised as it is pumped into each well. Before running the gel, add an additional 4 μl of ethidium bromide into the TBE at the cathode end of the gel box.

Be sure that the end of the gel with the wells is toward the anode and the bottom of the gel is positioned toward the cathode. The gel should be run using 90 volts for *ca* 30 min, allowing for the tracking dye to reach the bottom of the gel.

DNA bands are then visualised using UV light. Based on the DNA ladder used, both the fragment size and quantity of DNA in the PCR product can be quantified using the position of the band in the gel and the brightness of each band, respectively.

- Preparation 1X TBE Electrophoresis Buffer

Tris (hydroxymethyl) aminomethane	10.8 g
Boric acid	5.5 g
0.5 M EDTA	2 ml
Distilled water	to 1000 ml

Dissolve 10.8 g of Tris base and 5.5 g of boric acid in 800 ml of distilled water, then add 2 ml of 0.5 M EDTA. Add distilled water to bring the solution to 1000 ml. Store at room temperature.

- Preparation 1% Ethidium bromide

Ethidium bromide	0.1 g
Distilled water	100 ml

Dissolve 0.1 g of ethidium bromide in 100 ml of distilled water. Mix by shaking or inversion. Store this in a light-protected bottle in the refrigerator. This represents the stock solution. Make a working stain by diluting 0.4 ml of the stock solution with 400 ml of distilled water. This can be stored at room temperature. Exercise caution when making ethidium bromide as it is mutagenic.

- Preparation DNA Tracking Dye (with Glycerol or Sugar)

Bromophenol Blue	25 mg
Glycerol	3 ml
Sucrose	4 g
Distilled water	to 10 ml

Dissolve 25 mg bromophenol blue in 7 ml of distilled water. Add 3 ml of glycerol or 4 g of sucrose and mix by vortexing. Store at 4°C.

ExoSAP-IT® purification of PCR product

For sequencing and further analysis of the product resulting from PCR, excess primers and dNTPs need to be removed. There are several options to do this, but we find ExoSAP-IT® fast and relatively inexpensive. Transfer 5 μl of PCR product into a new 0.2 ml PCR tube and then add 2 μl of ExoSAP-IT® for a total volume of 7 μl. Place this tube in the thermal cycler and have it run the following programme:

Steps	*Step instructions*
1. Enzyme activation	Incubate at 37°C for 15 min
2. Enzyme denaturation	Incubate at 80°C for 15 min
3. Cycle termination	Incubate at 4°C forever

The resulting PCR product is free of excess nucleotides and primer and is now ready for further analysis (sequencing, SNP analyses, *etc.*). ExoSAP-IT® and cleaned PCR product should be stored at −20°C.

Big Dye cycle sequencing

This technique is used to determine the nucleotide sequence of genes amplified using polymerase chain reaction. It is very similar to PCR but utilises chain terminating, fluorescently labelled ddNTPs. Reagents should be stored at −20°C and thawed on ice:

5X Buffer (commercially available Big Dye comes with its own specific buffer) Big Dye
DNA Primers (only one direction per reaction; forward or reverse only)
Ultrapure water
Purified PCR product

Once the reagents have completely melted, they are combined in a 1.5 ml Eppendorf tube in the following order and quantities, forming the reaction mixture also known as the master mix. This step avoids

pipetting minute amounts into multiple individual tubes:

Reagent	1 Reaction	10 Reactions	x Reactions
Ultrapure water	6.4 μl	64 μl	$6.4 \times x$ μl
5X Buffer	2.1 μl	21 μl	$2.1 \times x$ μl
Primer (forward or reverse)	1.0 μl	10 μl	$1.0 \times x$ μl
Big Dye	0.5 μl	5.0 μl	$0.5 \times x$ μl
Total Master Mix Volume	10 μl	100 μl	$10 \times x$ μl

To allow for pipetting error, it is recommended that one extra reaction worth of reagents be added for every ten reactions needed. The master mix should be mixed by pump mixing, flicking the tube or gently inverting.

Transfer 10 μl of reaction mixture into a PCR tube for each DNA template and primer direction. In order to obtain sequence for the forward and reverse directions for each sample, two reactions must be done, one with the forward primer and one with the reverse.

Add 2 μl of cleaned PCR product to each 0.2 ml PCR tube for a total reaction volume of 12 μl. Load tubes into thermal cycler. For Big Dye cycle sequencing reactions use the following sequencing parameters:

Steps	*Step instructions*
1. Initial denaturation	Incubate at 95°C for 10 min
2. Denaturation	Incubate at 94°C for 1 min
3. Annealing	Incubate at 50°C for 1 min
4. Elongation	Incubate at 72°C for 2 min
5. Cycling step	Cycle to step 2 for 39 more times
6. Final elongation	Incubate at 72°C for 10 min
7. Cycle Termination	Incubate at 4°C forever

Once the thermal cycler reaches step 7, the cycle can be stopped and the tubes with sequencing product removed. Sequencing product should be stored at -20°C or -80°C and should always be kept on ice when in use.

Cycle sequencing Sephadex™ clean-up

Sequencing reactions must be completely cleaned before submitted for electrophoresis. Much like PCR product, the extra primers and dNTPs must be removed from the reaction mix while preserving the

sequenced target gene. This can be done by column purification using Sephadex™ G-50 Fine. The column is set up in a 96-well MultiScreen™ Millipore filter plate. This is done using a Sephadex™ loading plate and filling it with Sephadex™ G-50 Fine. The column is hydrated using 300 μl of distilled water and allowed to set up at room temperature for 20 min. Excess water is pulled from the column using centrifugation. The MultiScreen™ Millipore filter plate is attached to a 96-well collection plate by a MultiScreen™ centrifuge alignment frame and then centrifuged for 4 min at *ca* 500 *g*. The collection plate is emptied and new sterile collection plate is attached. Before adding the 12 μl sequencing reaction product to the column, 15 μl of distilled water is added to the sequencing product, adding volume. The now diluted 27 μl sequencing reaction mixture is added to one well of the 96-well MultiScreen™ Millipore filter plate where the Sephadex™ columns have been set up. This is then pulled through the column by centrifuging the plate at *ca* 500 *g* for 4 min. The collection plate is now ready for electrophoresis.

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